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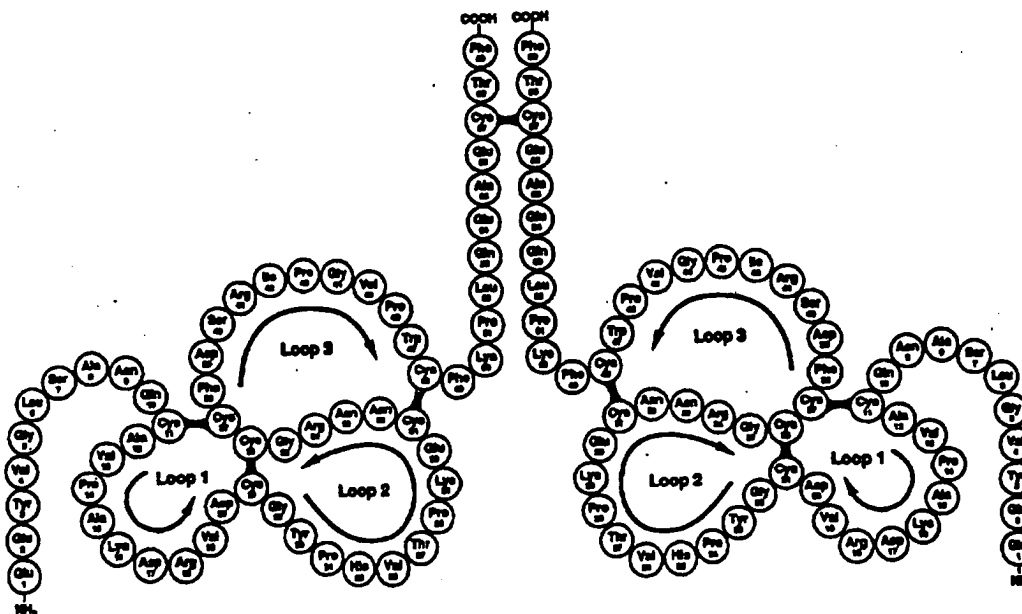
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(54) Title: TREFOIL PEPTIDE DIMER



(57) Abstract

A trefoil peptide containing a single trefoil domain characterized by being in dimer form.

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TREFOIL PEPTIDE DIMER

FIELD OF INVENTION

The present invention relates to trefoil peptide dimers, a method of preparing dimers of trefoil peptides, a pharmaceutical composition comprising trefoil peptide dimers and
5 the use thereof in the treatment of gastrointestinal disorders.

BACKGROUND OF THE INVENTION

Trefoil peptides form a family of peptides found mainly in association with the gastrointestinal tract. Mammalian trefoil peptides contain one or more characteristic trefoil domains (Thim et al., 1989) each of which is made up of a sequence of 38 or 39
10 amino acid residues in which 6 half-cystine residues are linked in the configuration 1-5, 2-4 and 3-6 thus forming a characteristic trefoil structure (Thim, 1989).

The mammalian trefoil peptides known at present contain either one or two trefoil domains (for review see Thim, 1994; Poulsom & Wright, 1993; Hoffmann & Hauser, 1993), whereas the frog, Xenopus laevis peptides and proteins containing one (Hauser
15 & Hoffmann, 1991) two (Hauser et al., 1992a) four (Hoffmann, 1988) or six (Hauser & Hoffmann, 1992b) trefoil domains have been described. The mammalian trefoil peptides containing one domain are the breast cancer associated pS2 peptide so far known from human (Jakowlev et al., 1984, Prud'homme et al., 1985) and mouse (Lefebvre et al., 1993) and intestinal trefoil factor so far known from human (Podolsky
20 et al., 1993; Hauser et al., 1993) and rat (Suemori et al., 1991; Chinery et al., 1992). Spasmolytic polypeptide (SP) which contains two trefoil domains has been described from man (Tomasetto et al., 1990), pig (Thim et al., 1982) and mouse (Tomasetto et al., 1990). In humans the three trefoil peptides hpS2, hITF and hSP, are all expressed under normal conditions in the gastrointestinal tract: hSP and hps2 in the epithelial
25 mucosal layer of the stomach (Tomasetto et al., 1990; Rio et al., 1988) and hITF in the epithelial mucosal layer of the small intestine and colon (Podolsky et al., 1993).

The physiological function of the trefoil peptides is not very well understood. Increased expression of trefoil peptides in the gastrointestinal tract has been reported in several conditions involving mucosal injury such as inflammatory bowel disease (Rio et al., 1991; Poulson et al., 1992; Wright et al., 1993) and ulceration in the stomach and 5 duodenum (Rio et al., 1991; Hanby et al., 1993; Wright et al., 1990). Consequently a mucosal repair function of the trefoil peptides has been suggested (e.g. Wright et al., 1993). Evidence of trefoil peptides promoting mucosal epithelial restitution after injury has recently been given by Dignass et al., 1994, Playford et al., 1994 and Babyatsky et al., 1994. The mechanism by which trefoil peptides promote their repair function may 10 be to cross-link mucin-glycoproteins to form a viscoelastic gel layer resistant to digestive enzymes (Thim, 1994; Gajhede et al., 1993).

The cloning of rat and human single-domain intestinal trefoil factor and the use of the intestinal trefoil factor in the treatment of gastrointestinal injury is described in WO 92/14837.

15 SUMMARY OF THE INVENTION

It has now been found possible to prepare dimers of trefoil factors which have only one trefoil domain and which have interesting pharmacological properties.

Accordingly, the present invention relates to a trefoil peptide containing a single trefoil domain, the peptide being characterized by being in dimer form.

20 As indicated above, trefoil peptides are believed to contribute to the healing of peptic ulcers and other mucosal injuries by stabilizing the mucus layer of the intestinal tract. The mechanism of this stabilization is at present unknown. However, the X-ray structure of porcine pancreatic spasmolytic polypeptide (PSP) (cf. Gajhede et al., 1993) which has two trefoil domains shows that the majority of conserved residues contribute 25 to a cleft, 8-10 Å wide, found in each of the trefoil domains. Preliminary docking experiments have shown that the cleft could accommodate part of an oligosaccharide chain, for instance the carbohydrate attached to a mucin glycoprotein. If this is the case,

PSP with two such clefts may cross-link mucins, helping them to form a protective gel over the mucosal epithelium. It is not known at present whether trefoil peptides with a single trefoil domain (such as ITF and pS2) form dimers in vivo to exert a similar function or whether they have a different mechanism of action. However, it is currently
5 believed that dimers of such trefoil peptides may indeed cross-link mucins and therefore be the active form of the peptides.

In another aspect, the present invention relates to a method of preparing a dimer of a trefoil peptide containing a single trefoil domain, the method comprising culturing a suitable host cell transformed with a DNA sequence encoding a trefoil peptide
10 containing one trefoil domain under conditions permitting production of the peptide, and recovering the resulting trefoil peptide dimer from the culture.

In a further aspect, the invention relates to a pharmaceutical composition comprising a dimer of a trefoil peptide containing a single trefoil domain together with a pharmaceutically acceptable diluent or vehicle.

15 In a still further aspect, the invention relates to a dimer of a trefoil peptide containing one trefoil domain for use as a medicament and the use of a dimer of a trefoil peptide containing one trefoil domain for the preparation of a medicament for the prophylaxis or treatment of gastrointestinal disorders.

DETAILED DESCRIPTION OF THE INVENTION

20 The dimer of the trefoil factor may, in particular, be a dimer of intestinal trefoil factor (ITF) or breast cancer associated peptide (pS2).

In particular, the trefoil factor is human ITF the monomeric amino acid sequence of which is

ZEYVGLSANQCAVPAKDRVDCGYPHVTPKEC~~NN~~RGCCFDSRIPGV~~W~~CFKP
25 LQEAECTF

↑
A

wherein Z is Glu, Gln or pyrGlu,

or a homologue thereof being capable of dimerization and exhibiting a similar activity;
or

human pS2 the monomeric amino acid sequence of which is

5 ZAQTETCTVAPRERQNCGFPGVTPSQCANKGCCFDDTVRGVPWCFYPNTID
VPPEEECEF

wherein Z is Glu, Gln or pyrGlu,

or a homologue thereof being capable of dimerization and exhibiting a similar activity.

Homologues of ITF or pS2 comprise the same cysteine pattern and disulphide
10 arrangement (Fig. 1) and exhibit a certain sequence homology (understood to mean
either identical amino acids in corresponding positions or conservative substitutions) in
loop 1, 2 and 3. The sequence homology in the loop regions may vary from 1 to 10
amino acid residues and the number of amino acid residues in each loop (apart from
the cysteines) may vary from 7 to 12, preferably from 9 to 10.

15 Homologues of ITF or pS2 may have one or more amino acid substitutions, deletions
or additions. These changes are preferably of such a nature that substitution does not
significantly affect the folding or activity of the protein. Small deletions are typically of
from 1 to about 3 amino acids in the loop regions and from 1 to about 10 amino acids
in the N- and C-terminal regions; single amino- or carboxyl-terminal extensions, such
20 as an amino-terminal methionine residue, a small linker peptide of up to about 10
residues, or a small extension that facilitates purification, such as a poly-histidine tract,
an antigenic epitope or a binding domain. See in general Ford et al., Protein Expression
and Purification 2: 95-107, 1991. Examples of conservative substitutions are within the
group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such
25 as glutamic acid and aspartic acid), polar amino acids (such as glutamine and

asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the present trefoil peptide and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244, 1081-1085, 1989). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g. mucosal healing, protection of mucosa, gastric ulcer healing) to identify amino acid residues that are critical to the activity of the molecule.

The homologue may be an allelic variant, i.e. an alternative form of a gene that arises through mutation, or an altered peptide encoded by the mutated gene, but having substantially the same activity as the present peptide. Hence mutations can be silent (no change in the encoded peptide) or may encode peptides having altered amino acid sequence.

The homologue of the present trefoil peptide may also be a species homologue, i.e. a polypeptide with a similar activity derived from another species, e.g. mouse, rat, rabbit, cow, pig or frog.

In a preferred embodiment, the trefoil peptide dimer of the invention has an approximate molecular weight of 13000. The dimer is composed of two trefoil peptide monomers linked by a disulphide bond between two cysteine residues in position 57 of ITF-like monomers or in position 58 of pS2-like monomers.

The trefoil peptide dimer is preferably produced by recombinant DNA techniques. To this end, a DNA sequence encoding the trefoil peptide may be isolated by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of

the peptide by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). For the present purpose, the DNA sequence encoding the peptide is preferably of human origin, i.e. 5 derived from a human genomic DNA or cDNA library.

The DNA sequence encoding the trefoil peptide may also be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage and Caruthers, Tetrahedron Letters 22 (1981), 1859 - 1869, or the method described by Matthes et al., EMBO Journal 3 (1984), 801 - 805. According to the phosphoramidite 10 method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The DNA sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202, Saiki et al., Science 239 (1988), 487 - 491, or Sambrook et al., *supra*.

15 The DNA sequence encoding the trefoil peptide is usually inserted into a recombinant vector which may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is 20 independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the trefoil peptide is operably linked to additional segments required for transcription of 25 the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g.

transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA encoding the trefoil peptide in mammalian cells are the SV40 promoter (Subramani et al., Mol. Cell Biol. **1** (1981), 854 -864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science **222** (1983), 809 - 814) or the adenovirus 2 major late promoter.

- 10 An example of a suitable promoter for use in insect cells is the polyhedrin promoter (US 4,745,051; Vasuvedan et al., FEBS Lett. **311**, (1992) 7 - 11), the P10 promoter (J.M. Vlak et al., J. Gen. Virology **69**, 1988, pp. 765-776), the *Autographa californica* polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immediate early gene 1 promoter (US 5,155,037; US 5,162,222), or the baculovirus 39K delayed-
15 early gene promoter (US 5,155,037; US 5,162,222).

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. **255** (1980), 12073 - 12080; Alber and Kawasaki, J. Mol. Appl. Gen. **1** (1982), 419 - 434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender
20 et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature **304** (1983), 652 - 654) promoters.

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. **4** (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the
25 gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae*

triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters. Suitable promoters are mentioned in, e.g. EP 238 023 and EP 383 779.

The DNA sequence encoding the trefoil peptide may also, if necessary, be operably
5 connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., Science 222, 1983, pp. 809-814) or (for fungal hosts) the TPI1 (Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or ADH3 (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) terminators. The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5 Elb
10 region), transcriptional enhancer sequences (e.g. the SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication.

15 When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2 μ replication genes REP 1-3 and origin of replication.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R.
20 Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include amdS, pyrG, argB, niaD or sC.

To direct a trefoil peptide of the present invention into the secretory pathway of the
25 host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the trefoil peptide in the

correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that normally associated with the peptide or may be from a gene encoding another secreted protein.

- 5 For secretion from yeast cells, the secretory signal sequence may encode any signal peptide which ensures efficient direction of the expressed trefoil peptide into the secretory pathway of the cell. The signal peptide may be naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the α -factor signal peptide (cf. US 4,870,008), the
- 10 signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., Nature **289**, 1981, pp. 643-646), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., Cell **48**, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast **6**, 1990, pp. 127-137).
- 15 For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and upstream of the DNA sequence encoding the trefoil peptide. The function of the leader peptide is to allow the expressed peptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e.
- 20 exportation of the trefoil peptide across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast α -factor leader (the use of which is described in e.g. US 4,546,082, US 4,870,008, EP 16 201, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a leader peptide not found in nature.
- 25 Synthetic leader peptides may, for instance, be constructed as described in WO 89/02463 or WO 92/11378.

For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal

peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α -amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. Suitable signal peptides are disclosed in, e.g. EP 238 023 and EP 215 594.

For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic hormone precursor signal peptide (cf. US 5,023,328).

The procedures used to ligate the DNA sequences coding for the trefoil peptide, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989).

The host cell into which the DNA sequence encoding the trefoil peptide is introduced may be any cell which is capable of producing the peptide in dimer form and includes yeast, fungi and higher eukaryotic cells.

15 Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10), CHL (ATCC CCL39) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. **159** (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. **1** (1982), 327 - 341; Loyter
20 et al., Proc. Natl. Acad. Sci. USA **79** (1982), 422 - 426; Wigler et al., Cell **14** (1978), 725; Corsaro and Pearson, Somatic Cell Genetics **7** (1981), 603, Graham and van der Eb, Virology **52** (1973), 456; and Neumann et al., EMBO J. **1** (1982), 841 - 845.

Examples of suitable yeasts cells include cells of *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or
25 *Saccharomyces kluyveri*. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides therefrom are described, e.g. in US 4,599,311, US 4,931,373, US 4,870,008, 5,037,743, and US 4,845,075, all of which are hereby

incorporated by reference. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4,931,373. The DNA sequence encoding the trefoil peptide may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable yeast cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., J. Gen. Microbiol. 132, 1986, pp. 3459-3465; US 4,882,279).

Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans* or *A. niger*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 238 023, EP 184 438. The transformation of *F. oxysporum* may, for instance, be carried out as described by Malardier et al., 1989, Gene 78: 147-156. The transformation of *Trichoderma* spp. may be performed for instance as described in EP 244 234.

When a filamentous fungus is used as the host cell, it may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome to obtain a recombinant host cell. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination.

Transformation of insect cells and production of heterologous polypeptides therein may be performed as described in US 4,745,051; US 4,879,236; US 5,155,037; 5,162,222; EP 25 397,485) all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. US 5,077,214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementioned references.

The transformed or transfected host cell described above is then cultured in a suitable nutrient medium under conditions permitting the expression of the trefoil peptide after which all or part of the resulting peptide may be recovered from the culture in dimer form. The medium used to culture the cells may be any conventional medium suitable
5 for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The trefoil peptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells
10 from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question.

15 In the pharmaceutical composition of the invention, the trefoil peptide dimer may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may be in a form suited for systemic injection or infusion and may, as such, be formulated with sterile water or an isotonic saline or glucose solution. The
20 compositions may be sterilized by conventional sterilization techniques which are well known in the art. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with the sterile aqueous solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate
25 physiological conditions, such as buffering agents, tonicity adjusting agents and the like, for instance sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

The pharmaceutical composition of the present invention may also be adapted for nasal, transdermal or rectal administration. The pharmaceutically acceptable carrier or
30 diluent employed in the composition may be any conventional solid carrier. Examples

of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Similarly, the carrier or diluent may include any sustained release material known in the art, such as glyceryl monostearate or glyceryl distearate, alone or mixed with a wax. The amount of solid carrier will vary widely but 5 will usually be from about 25 mg to about 1 g.

The concentration of the trefoil peptide in the composition may vary widely, i.e. from from about 5% to about 100% by weight. A preferred concentration is in the range of 50-100% by weight. A unit dosage of the composition may typically contain from about 1 mg to about 200 mg, preferably from about 25 mg to about 75 mg, in particular about 10 50 mg, of the peptide.

As indicated above, the trefoil peptide dimer of the invention believed to be the active form of the peptide. As such it is contemplated to be advantageous to use for prevention or treatment of gastrointestinal disorders. More specifically, it is contemplated for use in the treatment of gastric or peptic ulcers, inflammatory bowel 15 disease, Crohn's disease or injury to the intestinal tract caused by radiation therapy, bacterial or other infections, etc. The dosage of the polypeptide administered to a patient will vary with the type and severity of the condition to be treated, but is generally in the range of 0.1-1.0 mg/kg body weight.

BRIEF DESCRIPTION OF THE DRAWINGS

20 The present invention is described in further detail in the example with reference to the appended drawings wherein

Fig. 1 Proposed structure of human intestinal trefoil factor ITF. The primary amino acid sequence is taken from Hauser et al., 1993, and the disulphide bonds are placed in homology to PSP and pS2 (Thim, 1988).

25 Fig. 2 Reversed phase HPLC on a Vydac 214TP54 column of supernatant from yeast strain HW⁺6 expressing rat ITF.

- Fig. 3 Ion exchange chromatography on a Fast Flow SP-Sepharose column of partially purified human ITF. The amount of hITF (monomer) and hITF (dimer) were determined by analytical HPLC. The bars indicate the fractions pooled for further purification of the monomer and dimer forms. The dashed line shows the concentration of NaCl in the eluting solvent.
- Fig. 4 Reversed-phase HPLC on a Vydac 214TP54 C4 column of purified rat ITF, dimer (A), human ITF (monomer) B, and human ITF (dimer) C. The dashed lines show the concentration of acetonitrile in the eluting solvent.
- Fig. 5 Reconstructed mass spectra of purified rat ITF (dimer) (A), human ITF (monomer) (B) and human ITF (dimer) (C).
- Fig. 6 Structure of the dimer form of human ITF.
- Fig. 7 Restriction map of plasmid KFN 1003.
- Fig. 8 Construction of plasmid pHW756.
- Fig. 9 Construction of plasmid pHW1066.

EXAMPLE

MATERIALS AND METHODS

Cloning of rat ITF (rITF) and human ITF (hITF)

The cloning of rat and human ITF was carried out as described in WO 92/14837 and as described by Suemori et al., 1991, and Chinery et al., 1992 (rat ITF), and Podolsky et al., 1993, and Hauser et al., 1993 (human ITF).

Construction of rITF and hITF expression plasmids

The expression plasmids pHW756 for secretion of rat ITF and pHW1066 for secretion of human ITF were constructed as outlined in Fig. 7-9. The yeast expression vector pKFN1003 (described in WO 90/10075) is a derivative of plasmid CPOT (Kawasaki, 5 G. International Conference on Yeast Genetics and Molecular Biology, Sept.17-24, 1984, Edinburgh, Scotland, Abstr. P15.). It has Schizosaccharomyces pombe TPI gene (POT) as selection marker (Russell, P.R., Gene 40 (1985), 125-130), and the S.cerevisiae triose phosphate isomerase (TPI) promoter and terminator for control of expression (Alber, T. and Kawasaki, G. J.Mol.Appl.Genet. 1 (1982), 419-434).

10 The rat ITF gene was initially cloned into Bluescript II KS(-) (Stratagene), from which it was propagated according to Fig. 8. The helper vector pSX54 providing useful cloning sites, is composed of pUC18 and pDN1050 (Diderichsen, B., Poulsen, G.B., Jørgensen, S.T. Plasmid 30 (1993), 312-315). The synthetic DNA linker Nco1-PfIM1 has the following sequence :

15 1858: 5'-CATGGCTGAAAGATTGGAAAAGAGACAAGAGTTCGTTGGTTTGT
CTCCATCCCAATGT-3' 58 bp

1862 : 5'-TTGGGATGGAGACAAACCAACGAACTCTTGTCTCTTTTCCAAT
CTTTCAGC-3' 51 bp

The linker codes for the C-terminal 8 amino acids of the leader sequence as described 20 in Thim, L., Norris, K., Norris, F., Nielsen, P.F., Bjørn, S., Christensen, M., Petersen, J. FEBS Lett. 318, (1993), 345-352, with a few changes in codon choice, and the N-terminal part of the rat ITF gene : QEFVGLSPSQC. The amino acid sequence of the signal and leader is as described *ibid.* :

MKAVFLVLSLIGFCWAQPVTGDESSVEIPEESLIIAENTTLANVAMAERLEKR.

The human ITF gene was cloned into PUC19 and propagated as described in Fig. 9. The synthetic DNA linker Nco1-BsaA1 has the following sequence :

2292 : 5'-CATGGCTGAAAGATTGGAAAAGAGAGAAGAATAC-3' 34 bp

2287 : 5'-GTATTCTTCTCTCTTTTCCAATCTTTCAGC-3' 30 bp

- 5 The linker codes for the C-terminal 8 amino acids of the leader as described for the rITF construction and for the N-terminal 3 amino acids of the hITF gene : EEY. The signal and leader are the same as above.

The expression plasmids were transformed into *S.cerevisiae* strain MT 663 (E2-7B X E11-36 a/α, Δtpi/Δtpi, pep 4-3/pep 4-3) by selection for growth on glucose as the sole carbon source.

The yeast transformants expressing rat ITF and human ITF were named HW756 and HW1066, respectively.

Fermentations

The transformants described above were cultivated at 30°C for 72h in yeast peptone dextrose (YPD) medium (Sherman et al., 1981) supplied with additional yeast extract (60 Gram/L). OD values at 660 nm of 153 and 232 for HW756 (rITF) and HW1066 (hITF), respectively, were reached at the end of the fermentations. The pH was adjusted to 2.5 with 1M phosphoric acid at the end of the fermentation, and the yeast cells were removed by centrifugation at 3000 rpm for 15 min.

20 Purification of recombinant rITF

The concentration of rITF in the yeast fermentation broth and fractions obtained during the purification was measured by analytical HPLC. Aliquots (usually 50-200 μL) were injected onto a Vydac 214TP54 reverse-phase C4 HPLC column (0.46 x 25

cM) equilibrated at 30°C at a flow rate of 1.5 mL/min. with 0.1% (v/v) TFA in 15% (v/v) acetonitrile. After 10 min of isocratic elution, the concentration of acetonitrile in the eluting solvent was raised to 55% over 40 min. Absorbance was measured at 214 nm. Three peaks eluting at 26.5 min., 27.3 min. and 28.2 min. (Fig. 2) was found to represent dimer forms of rITF. The peptides were quantified using a calibrated hSP standard (Thim et al., 1993).

The expression level for recombinant rat ITF in the present yeast system was 113 mg/L.

10 From a 10 L fermenter, 8.7 L of fermentation broth was isolated by centrifugation. The supernatant was diluted with 14.8 L of distilled water to lower the conductivity. The sample was pumped onto a Fast Flow S-Sepharose (Pharmacia) column (5 x 42 cM) with a flow rate of 600 mL/h. Prior to the application, the column was equilibrated in 50 mM formic acid buffer, pH 3.7. Rat ITF was eluted from the
15 column by 50 mM formic acid, pH 3.7 containing 50 mM NaCl. Fractions of 100 mL were collected at a flow rate of 600 mL/h and analysed for the content of rITF. Fractions from the previous step containing rITF were pooled (2.3 L) and pumped onto an Amberchrome (G-71) column (5 x 10 cM). Prior to the application, the column was equilibrated in 10 mM ammonium acetate buffer, pH 4.8, at a flow rate
20 of 0.5 L/h. After application, the column was washed with 0.5 L of equilibration buffer and eluted with 10 mM ammonium acetate buffer pH 4.8 containing 60% (v/v) of ethanol at a flow rate of 0.1 L/h. Fractions of 10 mL were collected and pooled according to their content of rITF. The ethanol concentration in the pool was increased from 60% (v/v) to 87% (v/v) by the addition of 2 volumes of ethanol
25 (99.9%, v/v) and rITF was precipitated by cooling to resulting mixture to minus 25°C for 16h.

The precipitate was collected by centrifugation for 1 h at 10,000 g at -25°C and redissolved at room temperature in 130 mL of 20 mM formic acid pH 3.0. The sample was pumped onto a Fast Flow SP-Sepharose (Pharmacia) column (5 x 20
30 cM) with a flow rate of 50 mL/h. Prior to the application, the column was equilibrated with 20 mM formic acid, pH 3.0. Peptides were eluted from the column

by a linear gradient between 1.5 L of 50 mM formic acid, pH 3.0 and 1.5 L of formic acid, pH 3.0 containing 0.5 M NaCl. Fractions (10 mL) were collected at a flow rate of 80 mL/h and the absorbance was measured at 280 nm. Fractions were assayed for the content of rITF. Fractions corresponding to rITF were pooled. Rat
5 ITF was further purified by preparative HPLC. Pooled fractions (900 mL) were pumped onto a Vydac 214TP1022 C4 preparative HPLC column (2.2 x 25 cm) equilibrated in 0.1% (v/v) TFA. The peptides were eluted at 25°C and at a flow rate of 5 mL/min. with a linear gradient (540 mL) formed from MeCN/H₂O/TFA (10:89.9:0.1, v/v/v) and MeCN/H₂O/TFA (65:34.9:0.1, v/v/v). UV absorption was
10 monitored at 280 nm and fractions corresponding to 10 mL were collected and analysed for the content of rITF. Fraction containing rITF were pooled and the volume reduced to 30% by vacuum centrifugation. From the resulting pool, rITF was isolated by lyophilization. The total yield of rITF from 8.7 L of fermentation medium was 236 mg corresponding to an overall purification yield of 24%.

15 Purification of recombinant hITF

The concentration of hITF in the yeast fermentation broth and fraction, obtained during the purification, was measured in an HPLC system identical to the one described for rITF. In this system, two peaks eluting at 21.2 min. and 27.1 min. were found by mass spectrometry and sequence analysis to represent a dimer and a
20 monomer form of hITF. The expression level for recombinant human ITF in the present yeast system was 90 mg/l.

From a 10 L fermenter, 8.0 L of fermentation broth were isolated by centrifugation. The sample was dialysed 3 times (each time in 24 h) against 40 L of 10 mM formic acid, pH 2.5. The sample was pumped (0.25 L/h) onto an SP-Sepharose Fast Flow
25 (Pharmacia) column (5 x 40 cm). The column was washed with 5 L of 20 mM formic acid, pH 2.5, and eluted with a linear gradient formed by 5 L of 20 mM formic acid, pH 2.5 and 5 L of formic acid, pH 2.5 containing 1 M of NaCl. Fractions of 100 mL were collected and analysed for the content of hITF (Fig. 3). Two forms of hITF were eluted from the column: one representing a monomer

form of hITF (eluting at 0.5 M of NaCl) and one representing a dimer form of hITF (eluting at 0.78 M of NaCl). Fraction corresponding to the two forms were pooled separately.

Each fraction was divided into three equal parts (volume: approx. 700 mL) and 5 pumped onto a Vydac 214TP1022 C4 column (2.2 x 25 cm) equilibrated in 0.1% (v/v) TFA. The peptides were eluted at a flow rate of 4 mL/min. with a linear gradient (540 mL) between MeCN/H₂O/TFA (10:89.9:0.1, v/v/v) and MeCN/H₂O/TFA (65:34.9:0.1, v/v/v). UV absorption was monitored at 280 nm and fractions corresponding to 10 mL were collected and analysed for the content of 10 hITF.

Fraction from the previous step containing hITF (monomer) and hITF (dimer) were pooled separately and the pH was adjusted to 3.0. The samples were applied separately onto an SP-Sepharose HiLoad 16/10 (Pharmacia) column (1.6 x 10 cm) equilibrated in 20 mM formic acid, pH 3.0 containing 40% (v/v) ethanol. The 15 column was washed with 80 mL of equilibration buffer and eluted at a flow rate of 4 mL/min. with a linear gradient between 200 mL of 20 mM formic acid, pH 3.0, 40% (v/v) ethanol and 200 mL of 20 mM formic acid, pH 3.0, 40% (v/v) containing 1 M NaCl. Fractions (5 mL) were collected and analysed for the content of hITF.

Fractions containing hITF (monomer) and hITF (dimer), respectively, were pooled 20 and the peptide content precipitated by adjusting the ethanol concentration to 90% (v/v) and cooling the mixture for 72h at -25°C. The precipitate was collected by centrifugation and lyophilized. The total yield from 8L of fermentation broth was 256 mg hITF (monomer) and 133 mg hITF (dimer) corresponding to an overall purification yield of 50% and 65%, respectively, for the monomer and dimer forms.

25 Characterization of recombinant rITF and hITF

After hydrolysis in 6 M HCl at 110°C in vacuum-sealed tubes for 24, 48 and 96 h, the samples (50 µg) were analysed on a Beckman (Model 121 MB) automatic amino

acid analyser. Half-cystine was determined as the S-B-(4-pyridylethyl) derivative, after reduction of the disulphide bonds by tributylphosphine (Rüegg & Rudinger, 1974), followed by coupling with 4-vinylpyridine (Friedman et al., 1970). Hydrolyses of 4-vinylpyridine-treated samples were performed by 4 M methanesulphonic acid or
5 3 M mercaptoethanesulphonic acid at 110°C for 24 h as described above. Amino acid sequence analysis was determined by automated Edman degradation using an Applied Biosystems Model 470A gas-phase sequencer (Thim et al., 1987).

Mass spectrometry analysis was performed using an API III LC/MS/MS system (Sciex, Thornhill, Ont., Canada). The triple quadrupole-instrument has a mass-to-
10 charge (m/z) range of 2400 and is fitted with a pneumatically assisted electrospray (also referred to as ion-spray) interface (Bruins et al., 1987; Covey et al., 1988). Sample introduction was done by a syringe infusion pump (Sage Instruments, Cambridge, MA) through a fused capillary (75 μ m i.d.) with a liquid flow rate set at 0.5-1 μ l/min. The instrument m/z scale was calibrated with the singly-charged
15 ammonium adduct ions of poly(propylene glycols) (PPGs) under unit resolution. The accuracy of mass measurements is generally better than 0.02%.

Fig. 4 shows the analytical HPLC chromatograms obtained on the purified rITF (Fig. 4A) and hITF (Fig. 4B and 4C). Recombinant rITF contains a mixture of 3 closely related peptides, and no attempts were made to separate these forms. When
20 analysed by electrospray mass spectrometry, three dominating molecular weights were found, corresponding to: 13112.2, 13096.6 and 13078.8 (Fig. 5A). The calculated molecular weight of rat ITF in a monomer form in which Cys-57 contains a free -SH group is 6558.3. The calculated molecular weight of rat ITF in a dimer form (e.g. an S-S bridge is established between two Cys-57) is 13114.6. From the
25 molecular weights found for the recombinant rat ITF it is clear that all three peptides represent dimer forms of rITF. From other trefoil peptides in which the N-terminal amino acid residue is Gln, e.g. PSP (Thim et al., 1985 and Tomasetto et al., 1990) it is known that this residue has a tendency to cyclize to form a pyrrolidone carboxylic acid (pyrGlu). In the case of rat ITF having a predicted N-terminal
30 sequence of Gln-Glu-Phe-Val-Gly, it seems reasonable to assume that the N-ter-

minal Gln could also cyclize to form pyrGlu. Such a derivatization would result in a decrease in the molecular weight of rat ITF (dimer) on 17 (one pyrGlu) or 34 (two pyrGlu) mass unit, respectively. The observed molecular weights on 13096.6 and 13078.8 (Fig. 5A) correspond to the dimer form of rat ITF in which one, respectively two, N-terminal Gln residues have cyclized. The calculated molecular weight of these forms are 13097.6 and 13080.6 being in good agreement with the experimentally determined values. Thus from the HPLC (Fig. 4A) and mass analysis (Fig. 5A) it is concluded that the recombinant rat ITF consists of 3 different dimer forms: one containing 2 N-terminal Gln, one containing 1 N-terminal Gln and 1 N-terminal pyrGlu and one containing 2 N-terminal pyrGlu. Table I shows the amino acid composition of rat ITF being in good agreement with the expected values.

Figs. 5A and 5B show the purity of hITF (monomer) and hITF (dimer), respectively as analysed by analytical HPLC. The dimer form (Fig. 5C) looks relatively pure whereas the monomer form (Fig. 5B) seems to be contaminated with material eluting in front of peptide. However, upon rechromatography of material eluting in the main peak, a similar chromatogram was obtained (results not shown). This seems to indicate an atypical behaviour of the hITF (monomer) on reverse phase columns rather than impurities. We have previously observed a similar behaviour of highly purified porcine PSP as well as highly purified recombinant hSP (Thim et al., 1993).

Mass spectrometry analysis of the hITF (monomer) shows a molecular weight of the dominating peak corresponding to 6694.0 (Fig. 5B). The molecular weight, as calculated from the amino acid sequence (Fig. 1), is 6574.4 assuming that Cys-57 exists on -SH form. The amino acid sequence analysis (Table II) shows the expected N-terminal sequence of Glu-Glu-Tyr-Val-Gly-. The amino acid composition analysis (Table I) shows the expected values except for the presence of 7.3 (8) cysteines. An additional cysteine linked to Cys-57 of hITF monomer would increase the molecular weight to 6694.7 which is very close to the value determined by mass spectrometry (6694.0). It is, therefore, assumed that in the hITF (monomer), Cys-57 is disulphide-linked to an additional cysteine. The minor molecular weight peak in the mass

spectrum (Fig. 5B) may represent another derivative of Cys-57 or may be an impurity in the preparation.

The calculated molecular weight of hITF (dimer), in which two monomers are linked by a disulphide bond between two Cys-57 residues, is 13146.8. This is in good agreement with the value determined by mass spectrometry (13147, Fig. 5C). The other peak in the mass spectrum (13169) probably represents the Na⁺ adduct of hITF (dimer). The sequence analysis (Table I) as well as the amino acid composition analysis (Table II) are also in good agreement with the expected values.

Table I

Amino acid composition of rat ITF (dimer), human ITF (monomer) and human ITF (dimer)

Amino acid		Rat ITF (dimer)		Human ITF (monomer)		Human ITF (dimer)	
5	Asx	11.92	(12)	6.00	(6)	12.01	(12)
	Thr ^a	8.00	(8)	2.03	(2)	4.01	(4)
	Ser ^a	9.86	(10)	2.04	(2)	4.01	(4)
	Glx	15.98	(16)	6.92	(7)	14.00	(14)
	Pro ^b	12.48	(12)	n.d. ^c	(6)	n.d. ^c	(12)
10	Gly	6.02	(6)	4.20	(4)	8.09	(8)
	Ala	2.04	(2)	3.91	(4)	7.90	(8)
	Val	11.92	(12)	4.92	(5)	9.86	(10)
	Met	1.80	(2)	0.00	(0)	0.00	(0)
	Ile	1.98	(2)	1.17	(1)	2.13	(2)
15	Leu	3.92	(4)	2.03	(2)	3.99	(4)
	Tyr ^b	2.02	(2)	1.94	(2)	3.92	(4)
	Phe	7.88	(8)	2.93	(3)	5.94	(6)
	Lys	2.14	(2)	3.07	(3)	6.09	(6)
	His	0.00	(0)	0.99	(1)	2.03	(2)
20	Trp	2.16	(2)	n.d. ^c	(1)	n.d. ^c	(2)
	Arg	3.94	(4)	2.96	(3)	6.05	(6)
	PE-Cys ^b	12.70	(14)	7.30	(7)	13.80	(14)
Total			(118)		(59)		(118)

Values in brackets are those deduced from cDNA: rat ITF (Chinery et al., 1992)
 25 human ITF (Hauser et al., 1993).

^a) Determined by extrapolation to zero time of hydrolysis

^b) Determined by hydrolysis in 4M methane sulphonic acid.

^c) Not determined

Table II

Automated Edman Degradation of rat ITF (dimer), human ITF (monomer) and human ITF (dimer)

5	Cycle No.	Rat ITF (dimer)		Human ITF (monomer)		Human ITF (dimer)	
		PTA-AA	Yield (pmol)	PTH-AA	Yield (pmol)	Yield (pmol)	
5	1	Gln	989	Glu	1517	Glu	2227
	2	Glu	731	Glu	1998	Glu	2361
	3	Phe	967	Tyr	2205	Tyr	2528
	4	Val	1072	Val	2409	Val	2431
	5	Gly	701	Gly	1625	Gly	1803
	6	Leu	838	Leu	2318	Leu	2253
	7	Ser	497	Ser	852	Ser	904
	8	Pro	965	Ala	1729	Ala	1712
10	9	Ser	406	Asn	1394	Asn	1518
	10	Gln	820	Gln	1494	Gln	1499
	11	(Cys)	n.d.	(Cys)	n.d.	(Cys)	n.d.
	12	Met	581	Ala	1243	Ala	1377
	13	Val	971	Val	1468	Val	1356
15	40	Pro	962	Pro	1223	Pro	1195
	15	Ala	529	Ala	1248	Ala	1249
	16	Asn	791	Lys	1270	Lys	1050
	17	Val	952	Asp	937	Asp	991
	18	Arg	331	Arg	891	Arg	964
20	19	Val	956	Val	1002	Val	1069
	20	Asp	476	Asp	847	Asp	932
	21	(Cys)	n.d.	(Cys)	n.d.	Cys	n.d.

Table II
Automated Edman Degradation of rat ITF (dimer), human ITF (monomer) and human ITF (dimer)

human ITF (dimer)							
Cycle No.	Rat ITF (dimer)		Human ITF (monomer)		Human ITF (dimer)		
	PTA-AA	Yield (pmol)	PTH-AA	Yield (pmol)	Yield (pmol)		
5	22	Gly	381	Gly	709	Gly	703
	23	Tyr	352	Tyr	894	Tyr	792
	24	Pro	927	Pro	766	Pro	701
	25	Thr	498	His	309	His	236
	26	Val	812	Val	755	Val	670
	27	Thr	510	Thr	505	Thr	576
10	28	Ser	225	Pro	458	Pro	473
	29	Glu	398	Lys	444	Lys	321
	30	Gln	499	Glu	219	Glu	304
	31	(Cys)	n.d.	(Cys)	n.d.	(Cys)	n.d.
	32	Asn	557	Asn	312	Asn	300
	33	Asn	591	Asn	464	Asn	503
	34	Arg	196	Arg	325	Arg	294
	35	Gly	222	Gly	239	Gly	223
15	36	(Cys)	n.d.	(Cys)	n.d.	(Cys)	n.d.
	37	(Cys)	n.d.	(Cys)	n.d.	(Cys)	n.d.
	38	Phe	335	Phe	189	Phe	174
	39	Asp	275	Asp	133	Asp	137
	40	Ser	164	Ser	49	Ser	43
20	R.Y.		97.0%		93.2%		92.5%

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CLAIMS

1. A trefoil peptide containing a single trefoil domain characterized by being in dimer form.
2. A peptide according to claim 1 which is intestinal trefoil factor (ITF).
- 5 3. A peptide according to claim 2 which is human ITF.
4. A peptide according to claim 2 which has an approximate molecular weight of 13000.
5. A peptide according to claim 2 wherein the ITF dimer is composed of two ITF monomers linked by a disulphide bond between two cysteine residues in position 57
10 of each monomer.
6. A peptide according to claim 1 which is breast cancer associated peptide (pS2).
7. A peptide according to claim 6 which is human pS2.
8. A peptide according to claim 7 wherein the pS2 dimer is composed of two pS2 monomers linked by a disulphide bond between two cysteine residues in position 58
15 of each monomer.
9. A method of preparing a dimer of a trefoil peptide containing a single trefoil domain, the method comprising culturing a suitable host cell transformed with a DNA sequence encoding a trefoil peptide containing one trefoil domain under conditions permitting production of the peptide, and recovering the resulting trefoil
20 peptide dimer from the culture.

10. A pharmaceutical composition comprising a dimer of a trefoil peptide containing a single trefoil domain together with a pharmaceutically acceptable diluent or vehicle.
11. A pharmaceutical composition according to claim 10 comprising a peptide
5 according to any of claims 2-8.
12. A dimer of a trefoil peptide containing one trefoil domain for use as a medicament.
13. Use of a dimer of a trefoil peptide containing one trefoil domain for the preparation of a medicament for the prophylaxis or treatment of gastrointestinal
10 disorders.

1/9

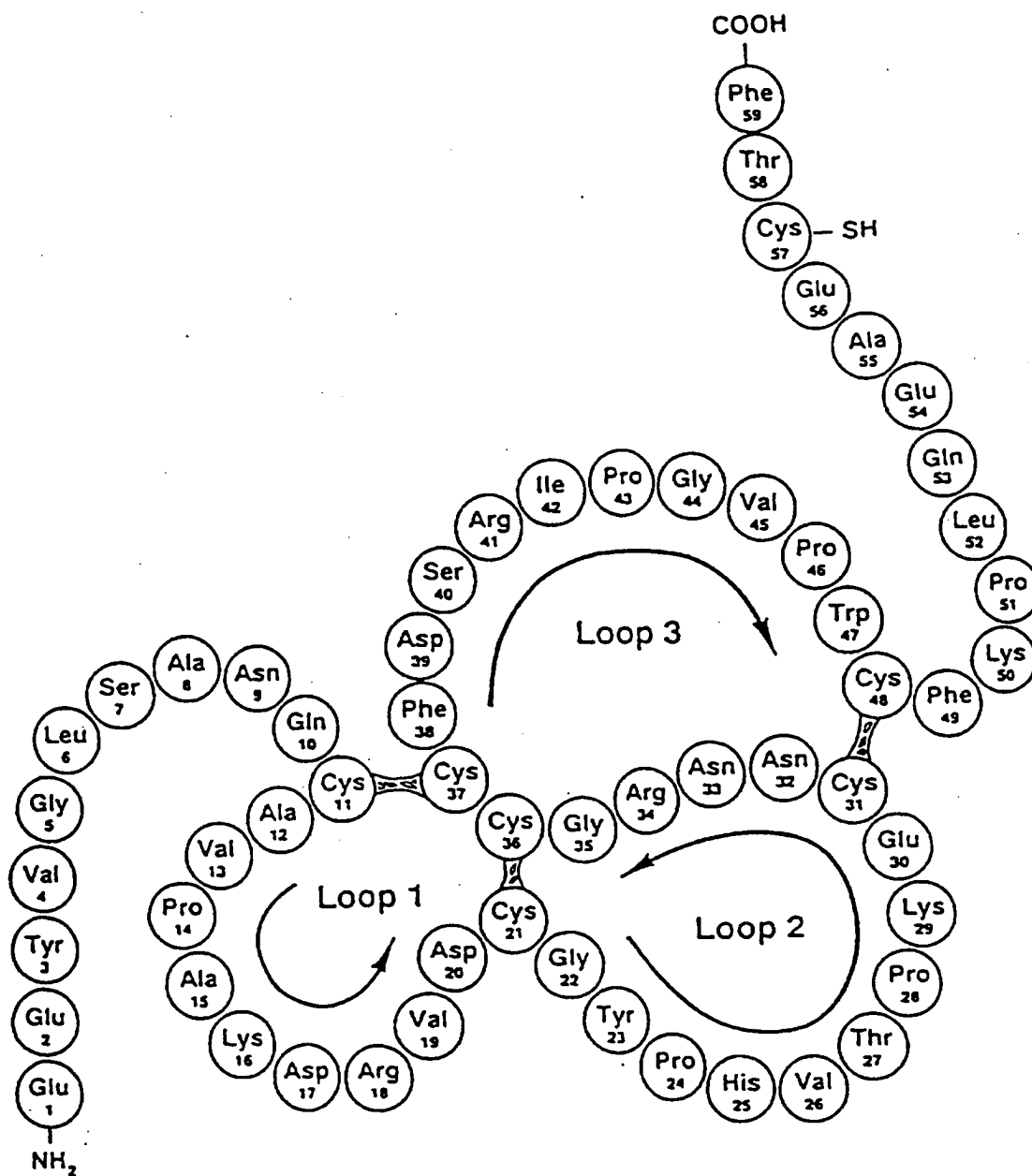


Fig. 1

2/9

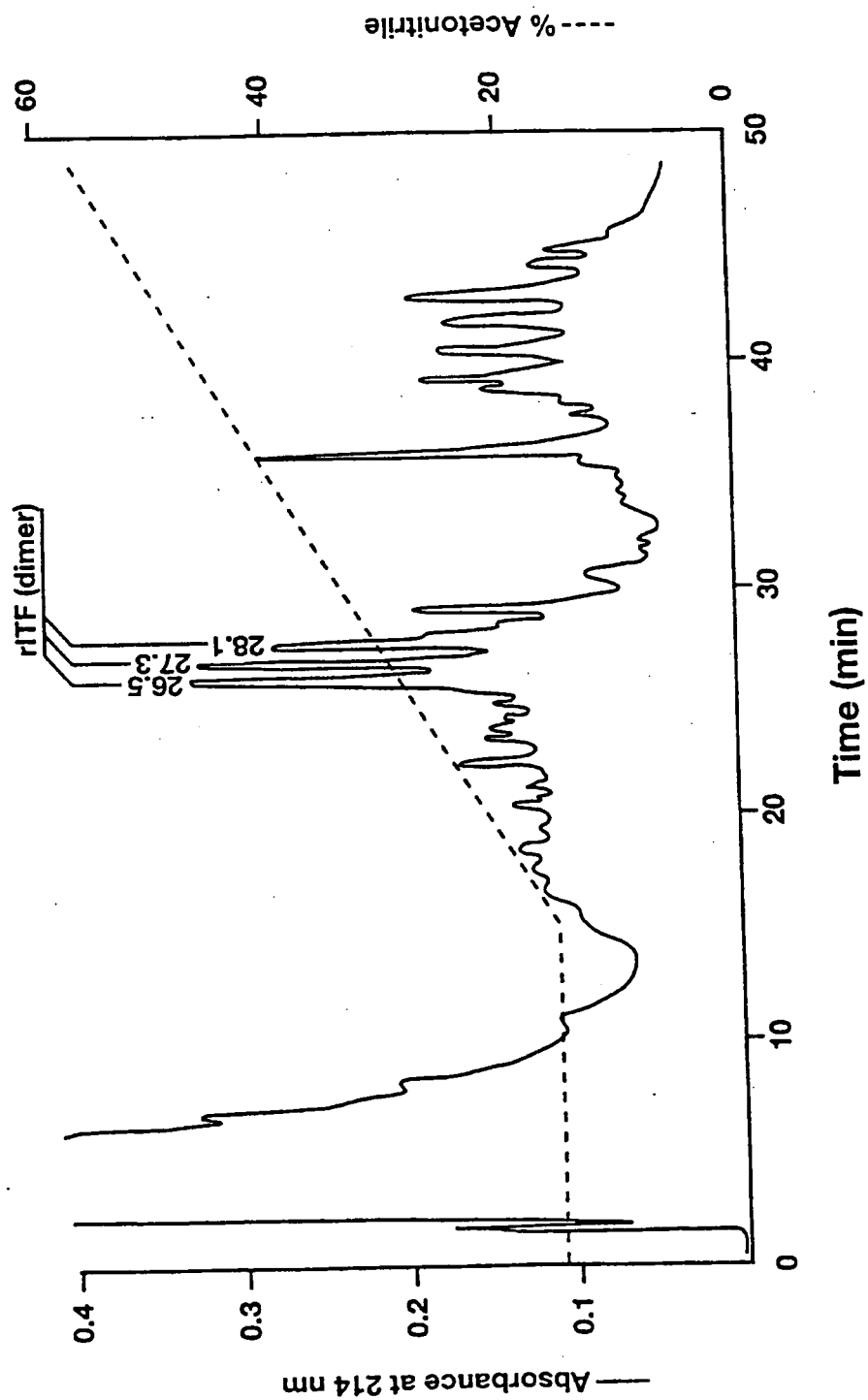


Fig. 2

3/9

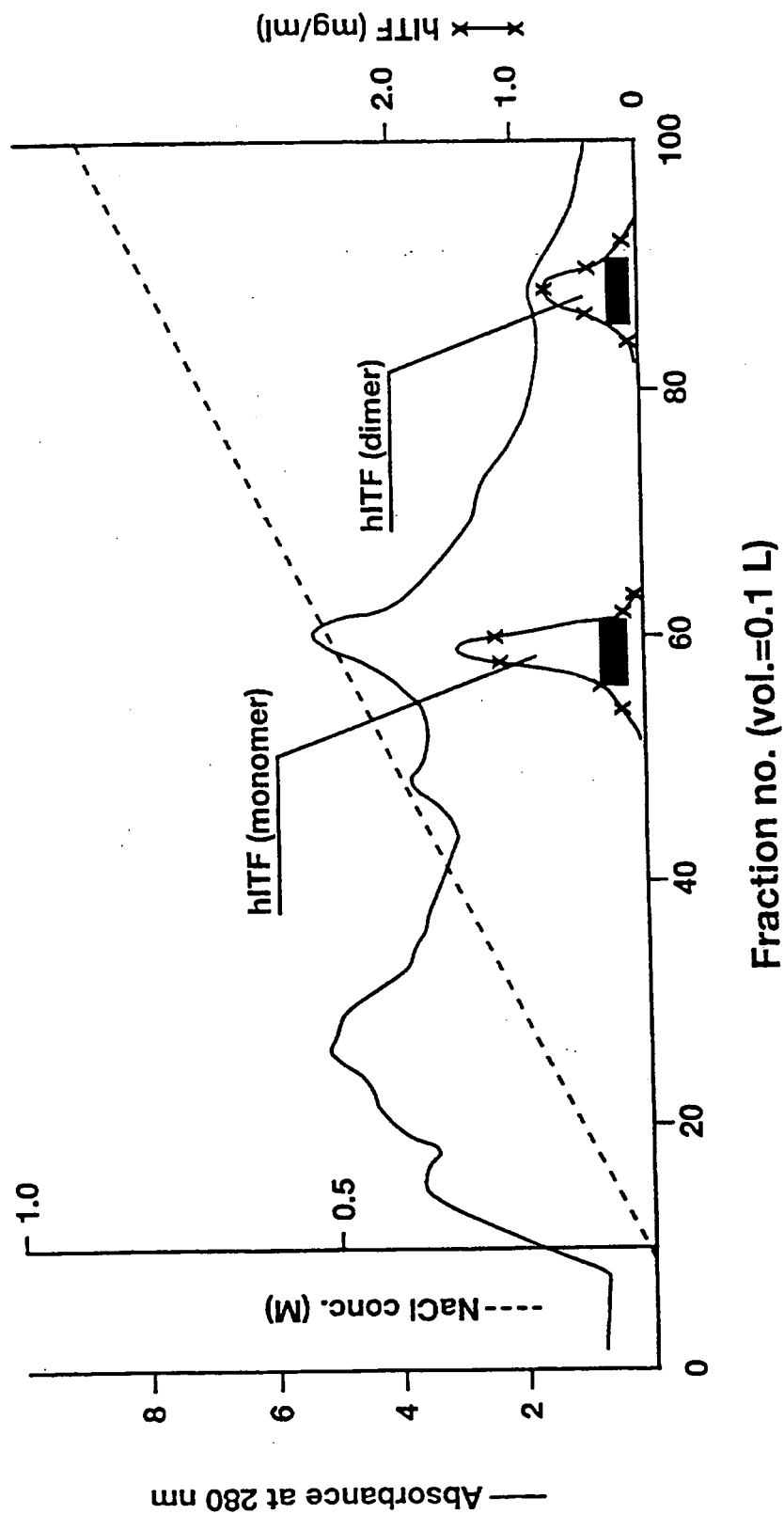


Fig. 3

4/9

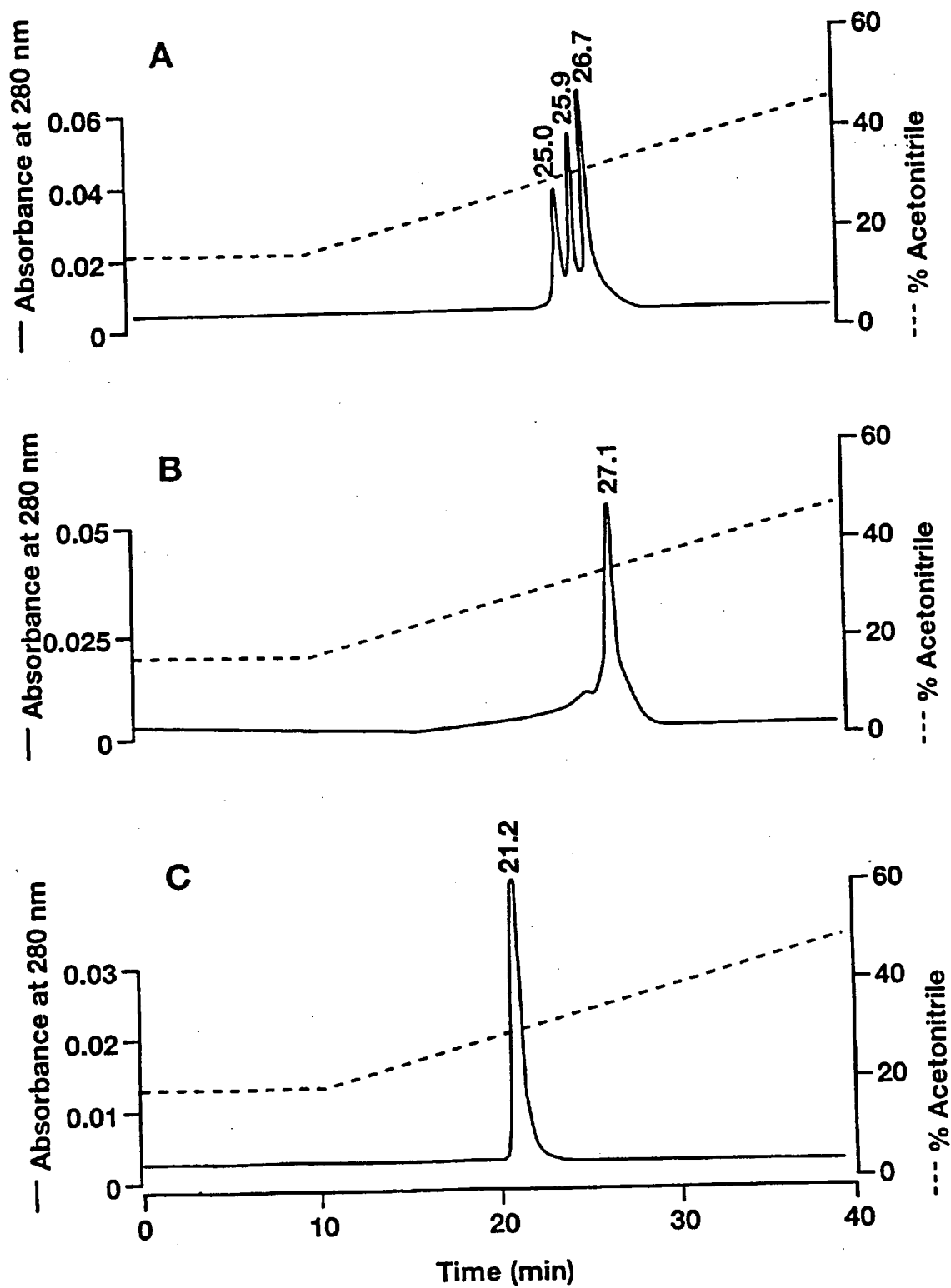


Fig. 4

5/9

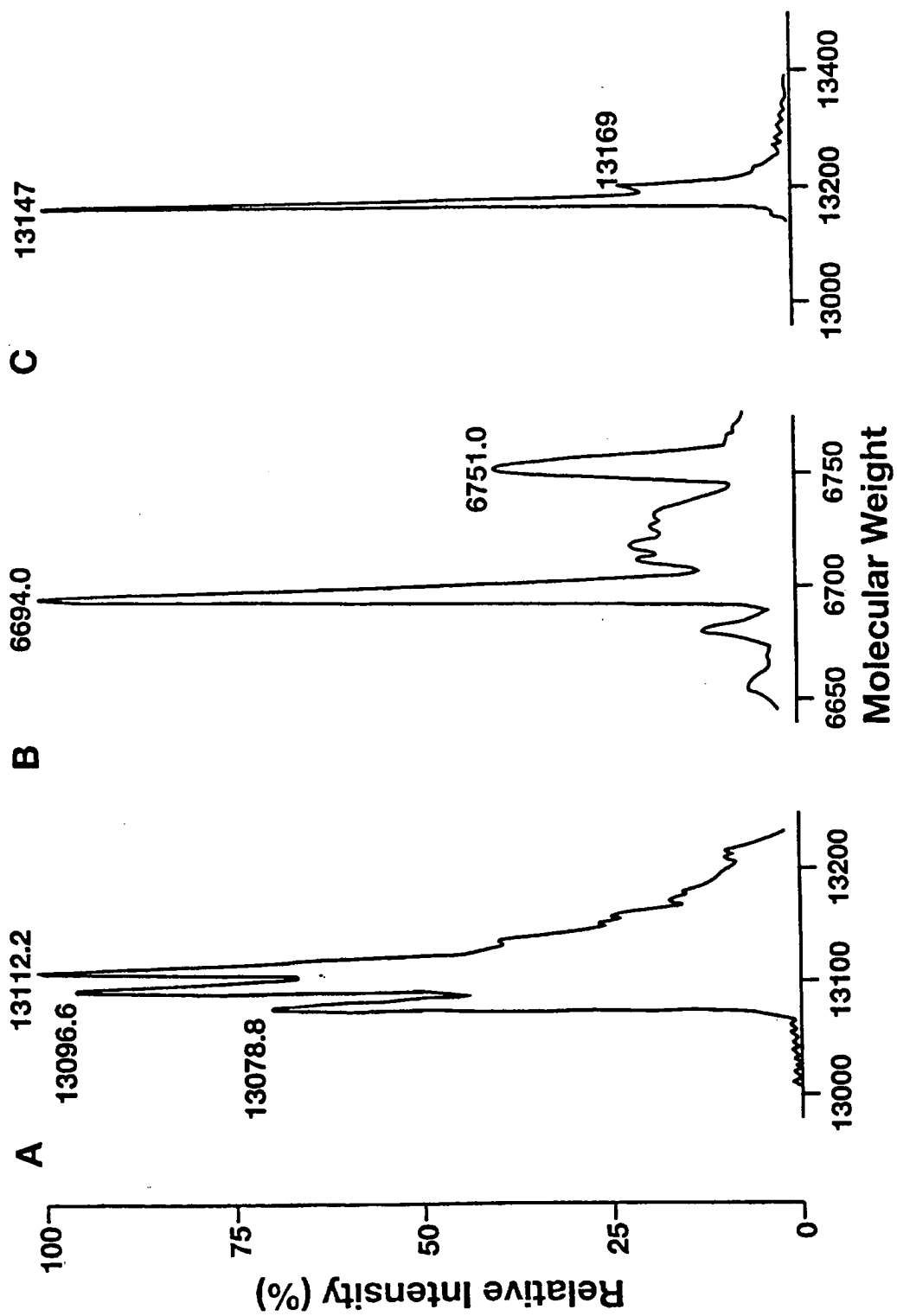
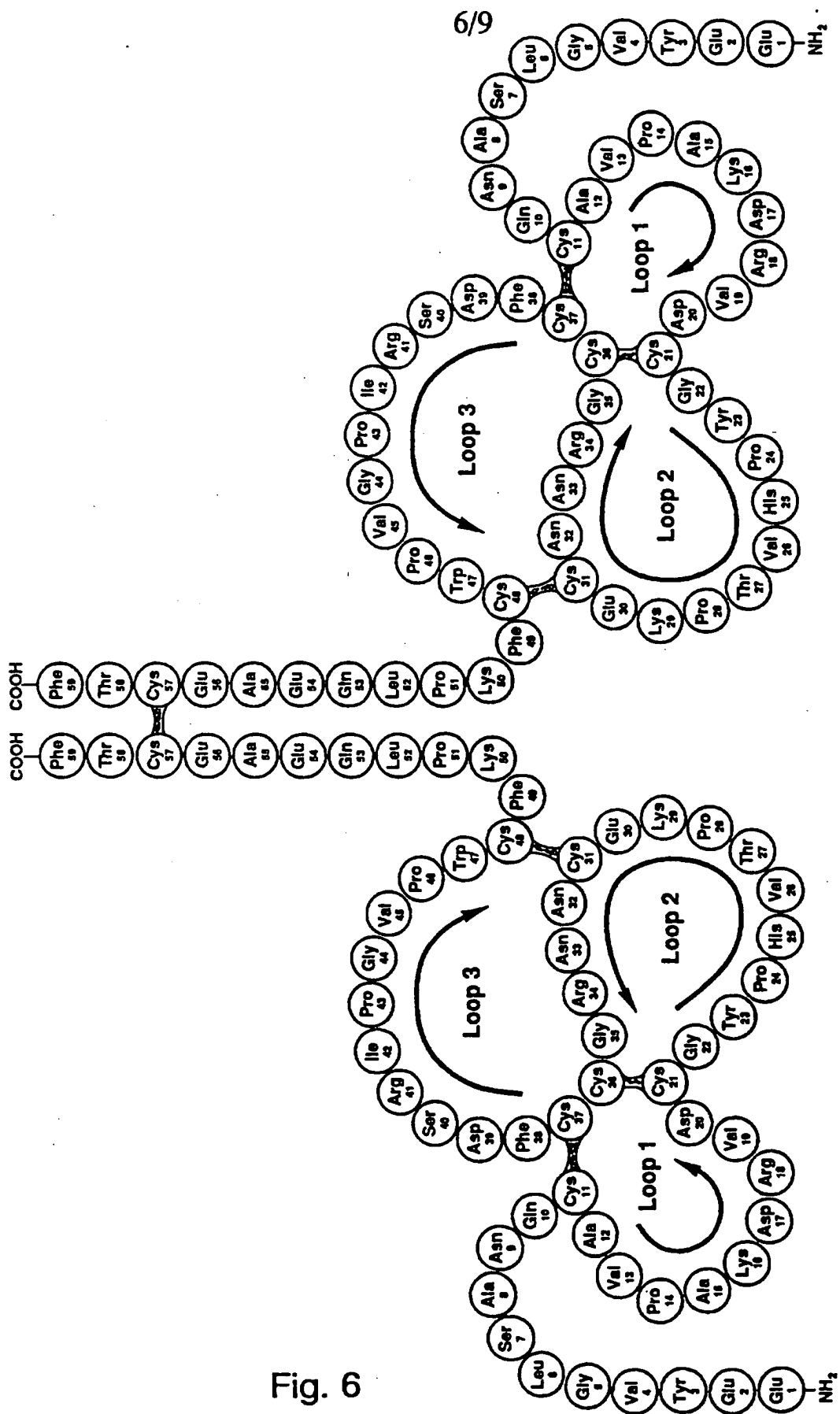
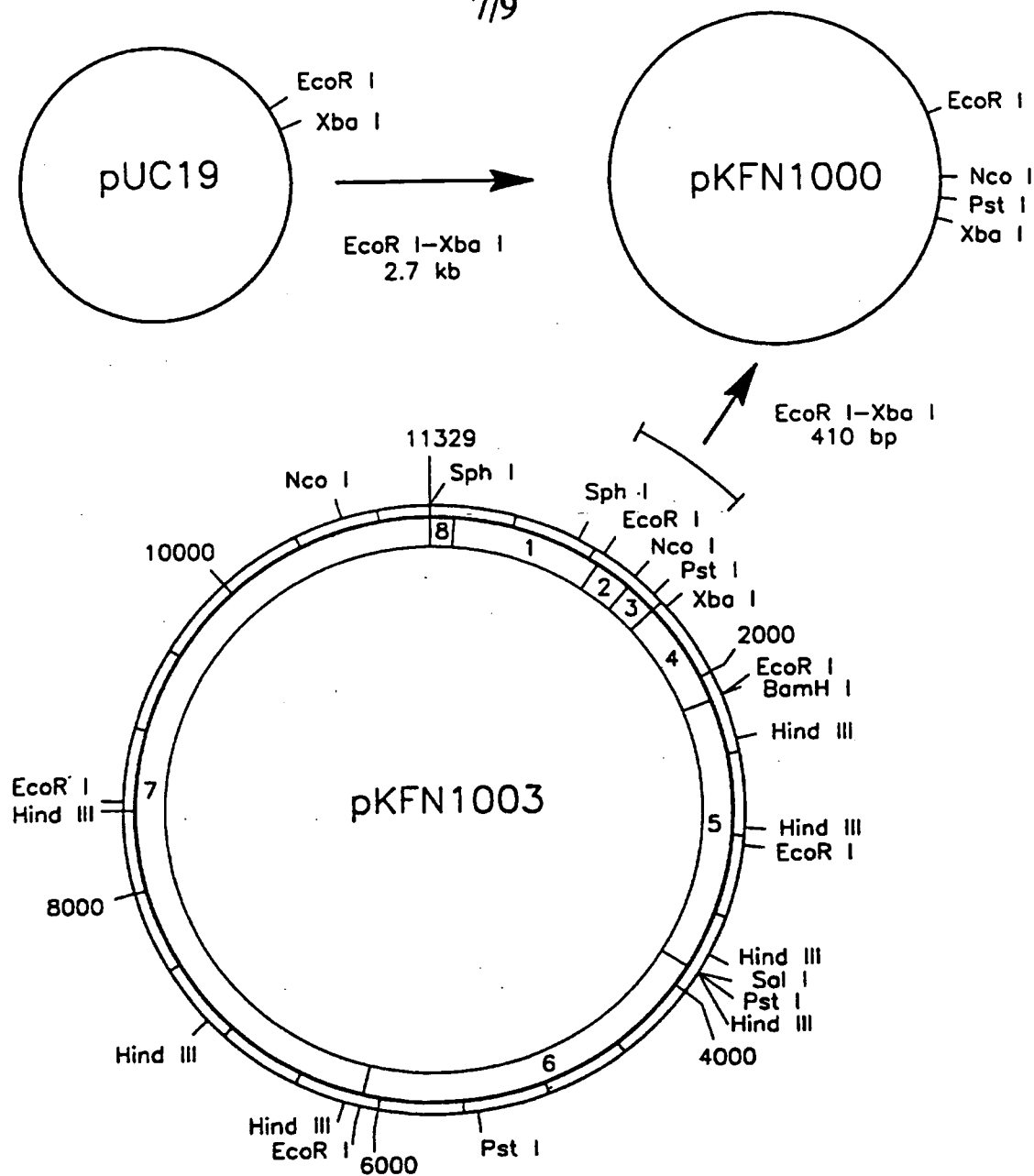


Fig. 5



7/9



1. TPI promoter from *S.cerevisiae*
2. Synthetic signal-leader gene
3. Synthetic aprotinine gene
4. TPI terminator from *S.cerevisiae*
5. TPI gene from *S.pombe*
6. Sequences from *E.coli* plasmids pBR322 and pUC13
7. Sequences from *S.cerevisiae* 2 μ plasmid
8. Linker sequence from *E.coli* plasmid pBR322

Fig. 7

8/9

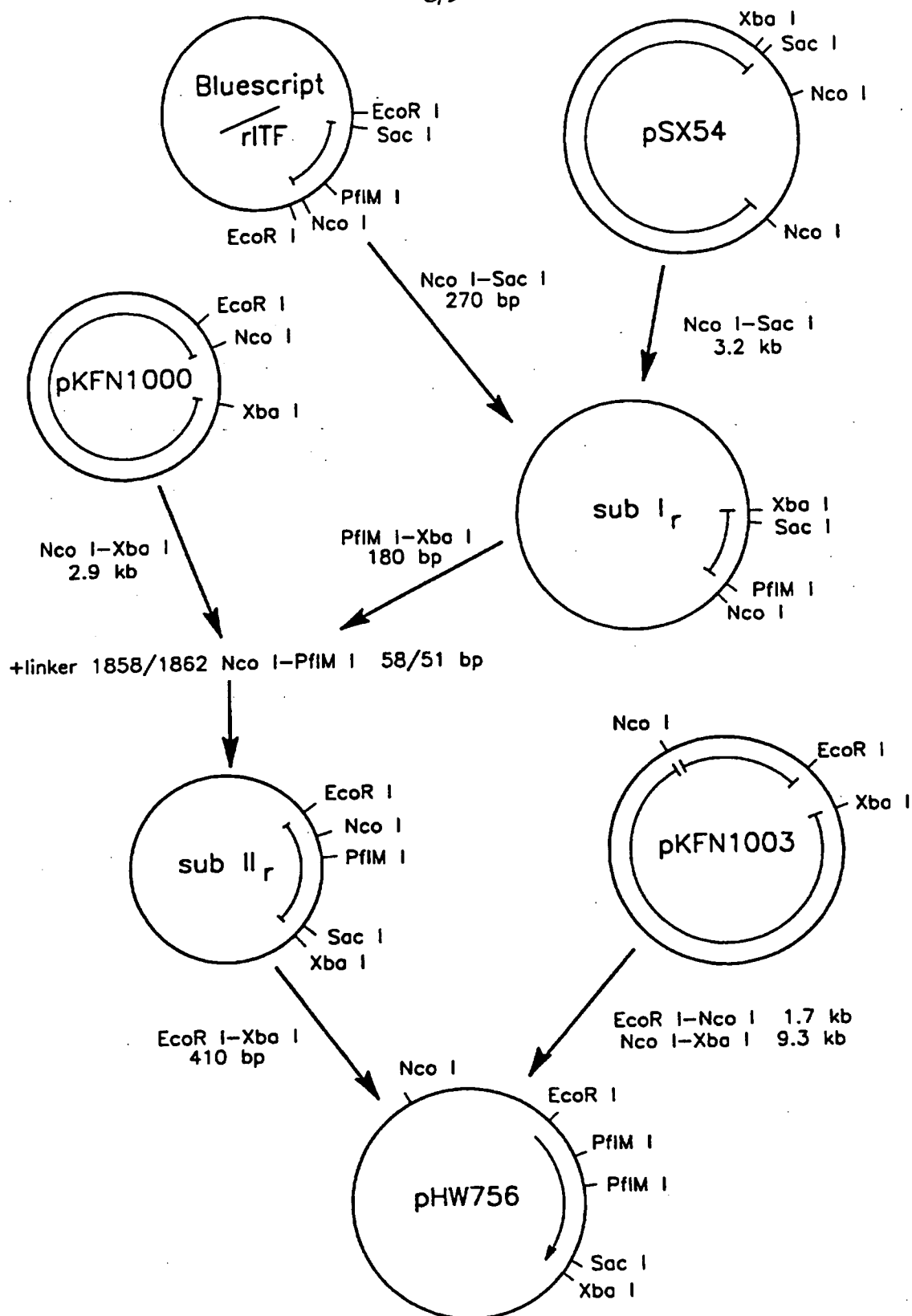


Fig. 8

9/9

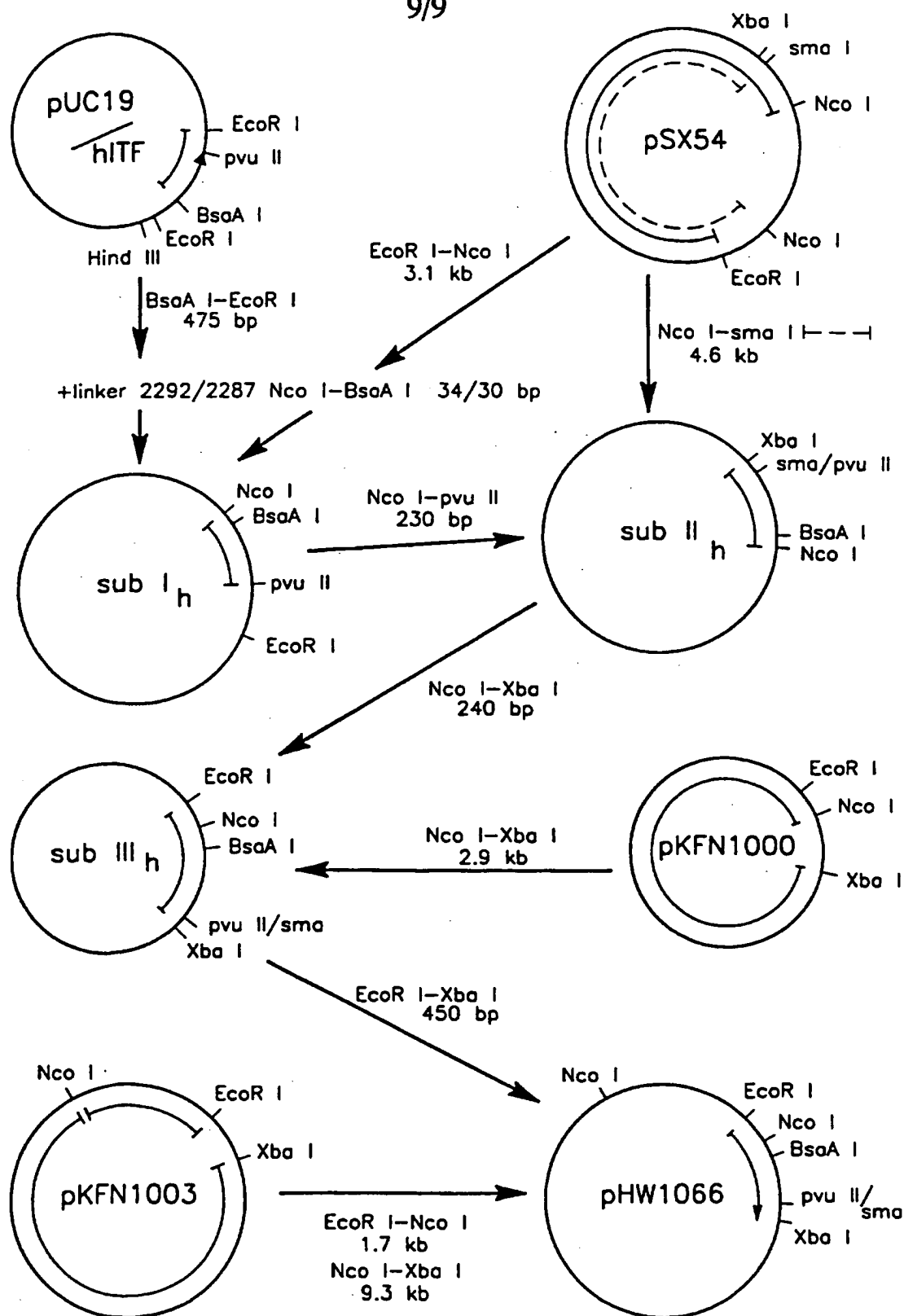


Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00343

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/575, A61K 38/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPI, IFIPAT, REG, CA, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	FEBS Letters, Volume 357, 1995, Rebecca Chinery et al, "Characterisation of the single copy trefoil peptides intestinal trefoil factor and pS2 and their ability to form covalent dimers" page 50 - page 54 --	1-13
P,X	Biochemistry, Volume 34, 1995, Lars Thim et al, "Characterization of Human and Rat Intestinal Trefoil Factor Produced in Yeast" page 4757 - page 4764 --	1-13
A	WO 9214837 A1 (THE GENERAL HOSPITAL CORPORATION), 3 Sept 1992 (03.09.92) -----	1-13

☐ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 December 1995

Date of mailing of the international search report

27 -12- 1995

Name and mailing address of the ISA/

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Information on patent family members

International application No.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9214837	03/09/92	AU-A- 1415892	15/09/92
		CA-A- 2104104	03/09/92
		EP-A, A- 0573544	15/12/93